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DOI: https://doi.org/10.1016/j.talanta.2021.122358

Talanta 232 (2021) 122358

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Simultaneous quantification of progestogens in plasma and serum by UHPLC-HRMS employing multiplexed targeted single ion monitoring

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ARTICLE INFO

Keywords:
Progesterone
Progestogen
Metabolites
Solid phase extraction
Single ion monitoring
LC-MS
Q Exactive

ABSTRACT

Progesterone is the predominant gestagen in most mammals studied so far. It plays a substantial role in the regulation of the female reproductive cycle and in providing support for pregnancy maintenance. Despite its known functions, gaps in knowledge are present regarding its reduced metabolites that potentially exert biological activity. Therefore, a new UHPLC-HRMS method based on a Q Exactive™ mass spectrometer was developed to detect and quantify simultaneously progesterone, its hormone precursor pregnenolone and 10 reduced progestogens (20α-DHP, 20β-DHP, 3α,5α-THP, 3α,5β-THP, 3β,5α-THP, 3β,5β-THP, 3α-DHP, 3β-DHP, 5α-DHP and 5β-DHP) in plasma and serum samples. Purification was achieved by an optimized solid phase extraction (SPE) and the analysis was conducted in positive electrospray ionization (ESI) mode with the application of multiplexed selected ion monitoring (msx-*t*-SIM). The method validation included the study of sensitivity, selectivity, curve fitting, carry-over, accuracy, precision, recovery and matrix effects. Despite the poor ionization properties of underivatized steroids, a high sensitivity in the range of pg/mL was achieved.

1. Introduction

Progesterone (P4) is a steroid hormone mainly synthesized by the corpus luteum in the female ovary, the placenta and the adrenal cortex [1]. Its physiology, production and mode of action have been studied thoroughly. P4 plays a key role in the maintenance of pregnancy [2] as well as during the development of mammary glands [3]. Additionally, its route of inactivation has been determined in detail [4].

The discovery of neuroactive steroids interacting with receptors and ion channels in the brain eventually widened the interest in unravelling the role of progesterone metabolites. A series of 3 alpha-hydroxy ring A-reduced pregnane steroids that own sedative, anxiolytic and anticonvulsant attributes such as allopregnanolone (5α-pregnan-3α-ol-20-one) or pregnanolone (5β-pregnan-3α-ol-20-one) belong to the category of neuroactive steroids. They bind with a high affinity and stereoselectively to γ-aminobutyric acid receptors ($GABA_A$) and are thus an important part of the central nervous system [5–7]. Furthermore, several reports were released on the correlation of 5α-dihydroprogesterone (5α-DHP) and 3α-dihydroprogesterone (3α-DHP) levels in the context of breast cancer [4,8,9]. Thereby, tumorous breast tissue was found to produce higher levels of 5α-DHP and decreased levels of 3α-DHP compared to healthy tissue. Whereas 5α-DHP promoted cell proliferation and detachment in cancerous tissue, 3α-DHP showed to have an opposite effect. In addition, first evidence was provided that 3α-DHP suppresses mitosis while stimulating apoptosis. Again, 5α-DHP acting as a counterpart exhibited the reverse impact. Blackmore [9] showed that various progesterone metabolites including epipregnanolone (5β-pregnan-3β-ol-20-one) and 20α-dihydroprogesterone (20α-DHP) were able to act as potent stimulators of free Ca^{2+} influx and intracellular Ca^{2+} mobilization in human platelets. Additionally, some 5α/β-reduced progesterone metabolites were reported to display further functions during pregnancy such as influencing the pain perception of mother and fetus as well as protecting nerve cells against hypoxic stress [11,12].

The progesterone metabolism illustrated in Fig. 1 shows the complexity of its reductions which primarily take place in the liver and the ovarian tissue [11,13–15]. Due to the two ketone groups and a Δ4-double bond, P4 is highly vulnerable to enzymatic reductions by reductases (5α- and 5β-reductase) and hydroxysteroid dehydrogenases (3α-, 3β-, 20α-, and 20β-hydroxysteroid dehydrogenase), reactions that are classified as phase I metabolisms. In non-conjugated reduced products, a theoretical vast number of 26 metabolites can be formed through

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https://doi.org/10.1016/j.talanta.2021.122358
Received 23 December 2020; Received in revised form 19 March 2021; Accepted 20 March 2021
Available online 8 April 2021

combination of all six enzymes. Of these, 18 have been already detected in blood, urine and/or faeces [14].

Since the 1970s, a substantial number of immunoassays was developed for the sensitive and specific detection of sexual steroids, one of the first being a radioimmunoassay (RIA) for 17β-estradiol by G. E. Abraham [15]. Immunoassays are inexpensive, fast, require small sample volumes and can be up-scaled for a high sample throughput but are also known to lack unique specificity due to potential cross-reactivity of the antibody with other similar-structured compounds [16–20]. In contrast, gas chromatography-mass spectrometry (GC/MS) as well as liquid chromatography-mass spectrometry (LC/MS) provide good linearity even down to low concentrations while also providing superior selectivity by chromatographic and mass-based separation of related steroid structures [18]. As steroids most often possess polar hydroxyl and ketone groups, a GC/MS analysis requires that these functional groups have to be replaced by derivatization to obtain sufficient volatility and thermal stability [19]. This labor-intensive preparation step can be omitted when working with LC/MS, allowing sensitive detection of the analytes by electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI). Using these methods, various steroid adducts such as $[M+H]^+$, $[M + NH_4]^+$, $[M + HCOO]^-$ or $[M+F]^-$ can be effectively generated [20].

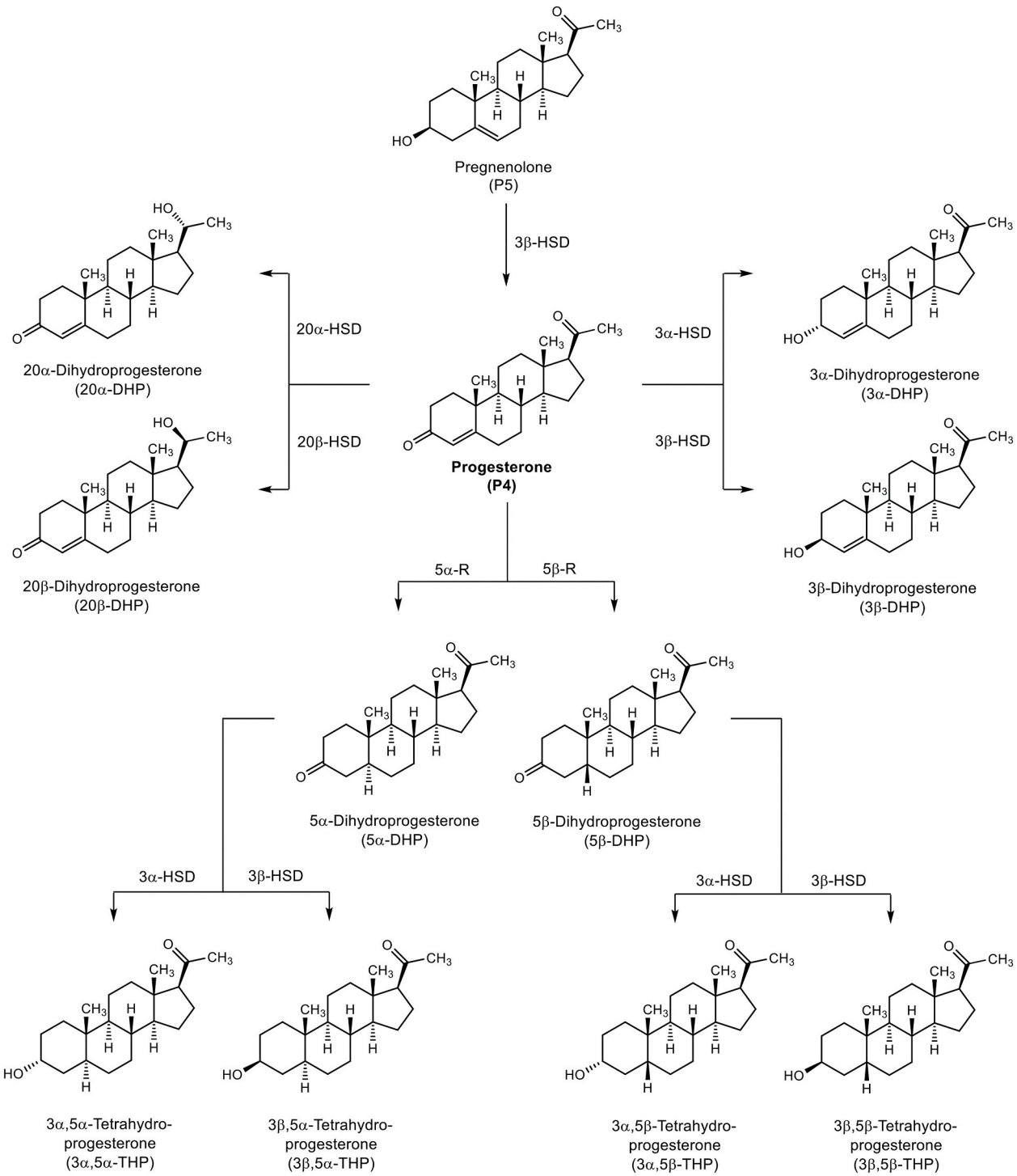

Fig. 1. First steps of the reductive biosynthetic pathway of progesterone into its metabolites (based on source [10,12,14] and [13]).

This study describes the establishment and validation of an UHPLC-HRMS method for the simultaneous detection and quantification of progesterone, its biological precursor pregnenolone and 10 reduced progesterone metabolites (20α-DHP, 20β-DHP, 3α,5α-THP, 3α,5β-THP, 3β,5α-THP, 3β,5β-THP, 3α-DHP, 3β-DHP, 5α-DHP and 5β-DHP) in plasma and serum samples (500 μL). Despite the poor ionization efficiency of steroids [21,22], we achieved a high sensitivity in the range of pg/mL without the need of derivatization. Selectivity was ensured by applying the high-resolution t-SIM (targeted single ion monitoring) mode. Research based on the quantification of large sample numbers is easily conductible due to the high throughput that we achieved by an efficient solid phase extraction protocol and a short chromatographic separation time.

2. Materials and method

2.1. Chemicals and reagents

Commercial steroid reference materials of 4-pregnen-3,20-dione (P4), 5-pregnen-3β-ol-20-one (P5), 5-pregnen-3β-ol-20-one-17,21,21,21-d4 (P5-*d4*), 5β-pregnan-3,20-dione (5β-DHP), 5α-pregnan-3,20-dione (5α-DHP), 5α-pregnan-3α-ol-20-one (3α,5α-THP), 5α-pregnan-3β-ol-20-one (3β,5α-THP), 5β-pregnan-3α-ol-20-one (3α,5β-THP), 5β-pregnan-3β-ol-20-one (3β,5β-THP), 4-pregnen-3α-ol-20-one (3α-DHP), 4-pregnen-3β-ol-20-one (3β-DHP), 4-pregnen-20α-ol-3-one (20α-DHP), 4-pregnen-20β-ol-3-one (20β-DHP) and 4-pregnen-3,20-dione-2,2,4,6,6,17α,21,21,21-d9 (P4-*d9*) were obtained from Steraloids (Newport, RI, USA). ULC-MS grade acetonitrile together with MeOH were from Biosolve BV (Valkenswaard, Netherlands). Formic acid (LC-MS grade) and *ortho*-phosphoric acid (85%, puriss p.a. grade) were supplied by Fluka (Buchs, SG, Switzerland). Ultrapure water (<2 ppb TOC) was produced using a Milli-Q® Advantage A10 water purification system by Merck (Bedford, MA, USA). Charcoal stripped bovine plasma of a domestic cattle collected 9 days postpartum was used as a surrogate matrix.

2.2. Standard solutions

Analytical standard solutions were stored at −20 °C until use. Stock solutions were prepared individually at 50 μg/mL by dissolving an accurate weight of the reference material in MeOH. Mixed working solutions were prepared in MeOH as a dilution series at the concentrations of 1000, 500, 50, 5.0 and 0.5 ng/mL of each steroid for the method optimization and the preparation of calibration and quality control samples. The internal standards (ISTD) P5-*d4* and P4-*d9* were mixed in an aqueous working solution at concentrations of 1 and 5 ng/mL, respectively.

2.3. Sample preparation

Plasma and serum samples were thawed at room temperature (22 °C). To prepare the samples for the calibration curve, an aliquot of the working standard solutions was spiked into charcoal stripped plasma to obtain the desired end concentration prior to the purification step. Quality control (QC) samples were prepared in the same fashion at three-levelled concentrations of choice (low, middle, high). For the evaluation of recovery and matrix effects, post-extracted blank plasma as well as neat solvent were spiked at three different concentrations covering the whole calibration range.

Following SPE protocol was developed on the basis of Strata-X (3 mL, 60 mg) polymeric reversed phase cartridges from Phenomenex (Torrance, CA, USA): Conditioning of the cartridges with 2 mL MeOH followed by equilibration with 2 mL H_2O; mixing 500 μL plasma with 500 μL of 4% aqueous H_3PO_4 and 1 mL aqueous ISTD (P5-*d4* and P4-*d9* at final concentrations of 5 and 1 ng/mL, respectively) solutions in a 5 mL Eppendorf tube; vortexed for 10 s. The sample mixture was loaded onto the sorbent and subsequently washed with 2 mL H_2O and an aqueous solution of 2 mL 40% MeCN/MeOH (85:15). Next, the sorbent was dried under vacuum (250–380 mm Hg) for 1 min. The steroids were then eluted using 2 mL 90% MeCN/MeOH (85:15), evaporated to dryness at 40 °C under a continuous flow of N_2 and reconstituted in 50 μL MeCN/H_2O 1:1 containing 0.1% formic acid. 5 μL of the final solution was injected for analysis. This protocol was also applied to all standard, QC and blank samples.

2.4. Chromatography

Liquid chromatography was performed on a Thermo Fisher UltiMate 3000 UHPLC (Waltham, MA, USA) build from a binary RS pump, an XRS open autosampler, a temperature-controllable RS column compartment. Sample separation was achieved at 25 °C on an ACQUITY UPLC HSS T3 column (2.1 × 100 mm, 1.8 μm particle size) protected by the corresponding HSS T3 VanGuard pre-column (2.1 × 5 mm, 1.8 μm particle size) and a Critical Clean ACQUITY guard filter (0.2 μm, 2.1 mm) from Waters (Milford, MA, USA). Eluent A consisted of H_2O and eluent B of acetonitrile, both acidified with 0.1% formic acid. The following gradient was applied at a constant flow rate of 450 μL/min: (i) 58% B isocratic from 0.0 to 0.5 min; (ii) linear increase to 62% B until 3.7 min; (iii) ramping to 66% until 6.0 min; (iv) switch to 100% B from 6 to 6.1 min; (v) holding 100% B until 10.0 min (vi) change until 10.1 min to the starting conditions of 58% B; (vii) equilibration for 3.9 min resulting in a total run time of 14 min.

2.5. Mass spectrometry

Mass spectra were acquired on a Thermo Fisher Scientific Q Exactive hybrid quadrupole-Orbitrap mass spectrometer (Waltham, MA, USA) equipped with a heated ESI source at position B and a voltage of 3.5 kV. Sheath, auxiliary and sweep gas (N2) flow rates were fixed at 45, 15 and 2 (arbitrary units), respectively. The capillary temperature amounted 250 °C and the auxiliary gas heater temperature was 450 °C. The S-lens RF level was set at 55.0. Multiplexed t-SIM in positive operation was chosen as the acquisition mode monitoring the most abundant ions of the investigated progestagens as listed in Table 1. The instrument was calibrated at a mass accuracy ≤ 2 ppm with a PierceTM LTQ Velos ESI Positive Ion Calibration Solution (Thermo). Further parameters were 35,000 full width at half maximum (FWMH) resolution at 200 *m/z*, a maximum IT of 57 ms, and an AGC target of 1e5. MS parameters were optimized by continuous flow injection (1 μL/min, Chemyx Fusion 101 HESI syringe pump, Chemyx, Stafford, TX, USA) of a solution containing a mixture of the progestogen standards (1000 ng/mL). Xcalibur 4.1 and TraceFinder 4.1 software (Thermo Fisher Scientific) were employed for data acquisition, peak-area integration and quantitation. A 15 ppm mass tolerance was set for peak detection.

2.6. Method validation

2.6.1. Selectivity

Chromatographic conditions were optimized and the targeted ions were selected with the mixed working solutions (see Chap. 2.2). Blank plasma and a calibration sample were injected and compared for the further investigation of interferences and selectivity. The minimum quality criteria required for the blank matrix used for QC and calibration sample preparation were signal responses below 20% of the limit of quantification (LOQ) for each analyte. Concerning the ISTD, a signal response under the LOD needed to be secured.

2.6.2. Carry-over

Blank samples (neat MeOH and blank plasma) were analyzed after the injection of a highly concentrated working standard solution (1000 ng/mL) in order to investigate possible carry-over. Again, carry-over in the blank samples should be lower than 20% of the signal response at the

Table 1
Chemical formula and monoisotopic masses of all targeted analytes as well as corresponding retention times, monitored ions and multiplexing count. (*coeluting compounds).

Compound	Formula	Neutral monoisotopic mass (u)	Monitored single ions (*m/z*)	Adduct	RT min	MSX count	ISTD
20α-DHP	$C_{21}H_{32}O_2$	316.2402	317.2475	$[M+H]^+$	2.61	1	P4-*d9*
20β-DHP	$C_{21}H_{32}O_2$	316.2402	317.2475	$[M+H]^+$	3.76	1	P4-*d9*
3α-DHP	$C_{21}H_{32}O_2$	316.2402	299.2369	$[M\text{-}H_2O + H]^+$	4.21	1	P5-*d4*
3β-DHP*	$C_{21}H_{32}O_2$	316.2402	299.2369	$[M\text{-}H_2O + H]^+$	3.52	1	P5-*d4*
5α-DHP	$C_{21}H_{32}O_2$	316.2402	317.2475	$[M+H]^+$	5.94	1	P5-*d4*
5β-DHP	$C_{21}H_{32}O_2$	316.2402	299.2369	$[M\text{-}H_2O + H]^+$	5.81	1	P5-*d4*
3α,5α-THP	$C_{21}H_{34}O_2$	318.2559	301.2526	$[M\text{-}H_2O + H]^+$	5.33	2	P5-*d4*
3α,5β-THP	$C_{21}H_{34}O_2$	318.2559	301.2526	$[M\text{-}H_2O + H]^+$	4.72	2	P5-*d4*
3β,5α-THP	$C_{21}H_{34}O_2$	318.2559	301.2526	$[M\text{-}H_2O + H]^+$	4.18	2	P5-*d4*
3β,5β-THP	$C_{21}H_{34}O_2$	318.2559	301.2526	$[M\text{-}H_2O + H]^+$	4.44	2	P5-*d4*
P4	$C_{21}H_{30}O_2$	314.2246	315.2319	$[M+H]^+$	3.92	3	P4-*d9*
P5*	$C_{21}H_{32}O_2$	316.2402	299.2369	$[M\text{-}H_2O + H]^+$	3.52	1	P5-*d4*
P5-d4	$C_{21}H_{28}D_4O_2$	320.2653	303.2478	$[M\text{-}H_2O + H]^+$	3.47	3	–
P4-d9	$C_{21}H_{21}D_9O_2$	323.2810	324.2883	$[M + H]^+$	3.85	4	–

LOQ and neglectable for the ISTD.

2.6.3. Curve plotting and sensitivity

For the quantification method, internal standards were added to the samples. The ratios of the peak areas of the analytes and their corresponding ISTDs were then plotted as a function of analyte concentration using least-squares linear regressions. A first set of calibrators (0.005, 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10, 15, 20, 30, 80, 240 ng/mL) was measured to determine the dynamic range, the limit of detection (LOD) and the limit of quantification (LOQ). The decision between linear and quadratic fitting was based on the obtained correlation coefficient R or R2 that defines the accuracy of the plotting. A fit was considered as acceptable if R or R2 were >0.99. Weighting functions were alike selected depending on the lowest bias. The LOD was reached at the analyte concentration which delivered a signal to background noise ratio higher than 3. For the specification of the LOQ, an S/N ratio of approximately 10 is required. For the quantification of authentic samples, the dynamic range was limited (0.02, 0.05, 0.1, 0.2, 0.5, 1, 5, 10 and 20 ng/mL) matching the relevant range of biological systems. P5-*d4* or P4-*d9* were chosen individually as an ISTD for each analyte depending on similarities in ionization behavior (see Table 1).

2.6.4. Accuracy and precision

Accuracy and precision were investigated using QC samples at three different concentrations. For the low QC sample, 0.06 ng/mL of P4, P5, 3β-DHP, 20α-DHP and 20β-DHP, 0.2 ng/mL of 5α-DHP, 5β-DHP, 3α-DHP, 3α,5α-THP, 3α,5β-THP, 3β,5α-THP and 0.4 ng/mL of 3β,5β-THP were used. 0.5 ng/mL and 0.2 ng/mL of every steroid was used for the middle and high QC, respectively. Each QC sample was analyzed in duplicate accompanied by a set of calibration samples. The determination of the intra-assay precision was performed in single runs, while inter-assay precision was derived from four independent measurements held on four different days. Both values were stated as relative standard deviations (RSD, %) and are a measure for repeatability and reproducibility. Accuracy, on the other hand, was calculated as a median relative error. Accuracy and precision were satisfactory when they were in the range of ±20% for the low QC and ±15% for the middle and high levels.

2.6.5. Recovery and matrix effect

Recovery and matrix effect in plasma were evaluated at three different concentrations for each analyte: 0.4 ng/mL (low), 6 ng/mL (middle) and 18 ng/mL (high) and by performing measurements in triplicate in two runs (n = 6). In total, three sample series had to be conducted: standards spiked into pure solvent, pre-extracted spiked samples and post-extracted spiked samples. In a first step, the recovery of extraction (RE) of each analyte had to be determined using equation (1) as analyte loss during sample preparation is not to be disregarded.

$$RE(\%) = \frac{C}{B} \times 100 \quad (1)$$

Thereby C corresponds to the peak area of the analyte recorded that was spiked into a blank plasma sample before the extraction, whereas B is considered to be the peak area at a recovery of 100% by spiking blank plasma after its extraction.

Matrix effects (ME), on the other hand, are caused by various compounds inside the plasma extract that have an impact on the quality of the measurement. The matrix effect of a compound is defined as

$$ME(\%) = \frac{B}{A} \times 100 \quad (2)$$

with A being the peak area of the standard analyte dissolved in mere reconstitution solution (50% acetonitrile + 0.1% formic acid). Thus, possible values bear the following meaning: <100% ≙ ion suppression, 100% ≙ no matrix effect and >100% ≙ ion enhancement.

3. Results and discussion

3.1. Method optimization

3.1.1. Development of a purification protocol

SPE was chosen as the purification technique in order to obtain interferent-free samples suitable for injection. Various types of cartridges were evaluated for the treatment of bovine plasma of a six-month pregnant cow. Among them were the Strata-X (30 mg, 1 mL and 60 mg, 3 mL) and the Strata-X-A (30 mg, 1 mL) from Phenomenex (Torrance, CA, USA) as well as the Oasis PRiME HLB (30 mg, 1 mL) from Waters. Different solvent mixtures based on H_2O, MeOH, MeCN, ethyl acetate and hexane were tested for optimization of the extraction conditions. The optimal procedure was to first dilute the plasma samples with 4% aqueous H_3PO_4 and, after loading and washing the SPE cardridges, elute the analytes with a mixture of H_2O/MeCN/MeOH. The lowest efficiency was obtained with the Strata-X-A, while the Strata-X and Oasis PRiME HLB had similarly high extraction efficiencies. However, the solubility of the Oasis PRiME HLB residue in MeCN/H_2O 1:1 was inferior to the Strata-X so that the Strata-X cartridge (60 mg, 3 mL) was finally chosen. The choice of reconstitution solvent was adjusted to the starting conditions of the chromatographic gradient.

3.1.2. Optimization of UHPLC-MS conditions

Working with heated (+)-ESI settings was of advantage as steroids were readily ionized to give adduct ions such as $[M+H]+$ and $[M\text{-}H_2O + H]+$, which led to high sensitivity. The parameters responsible for the sensitivity of the MS, such as gas flow, ion source temperature and voltage, were optimized using a steroid standard mixture (1 μg/mL) injected at a flow rate of 10 μL/min. Using a targeted single ion

monitoring (t-SIM) approach gave us the most sensitive detection mode for the steroids after also testing the options of PRM (parallel reaction monitoring) and full scan acquisition. The targeted ions were selected based on the most intense signal of each compound in the full scan mode.

Several UHPLC columns were evaluated to obtain the best chromatographic separation. Thereby, the Waters ACQUITY UPLC HSS T3 column (2.1 × 100 mm, 1.8 μm particle size) yielded the best peak shapes, the highest signal response as well as the greatest steroid separation power in the shortest time in comparison to Waters ACQUITY UPLC BEH C18 (2.1 × 100 mm, 1.8 μm particle size) and ACQUITY UPLC CSH C18 (2.1 × 100 mm, 1.8 μm particle size) columns. MeCN and H_2O as eluents provided low backpressure throughout all measurements, and 0.1% formic acid was chosen as additive due to its superior ionization efficiency of progestagens compared to ammonium formate and ammonium fluoride. A column temperature of 25 °C gave the best chromatographic separation, while higher temperatures promoted co-elution. The flow rate was optimized to give the best resolution at the highest possible flow rate of 0.45 mL/min. Finally, the elution gradient was adjusted to obtain the optimal separation of the analytes.

3.2. Method validation

3.2.1. Selectivity

The criteria for selectivity were chromatographic retention times, high-resolution separation as well as accurate masse detection of the selected ions. The retention time of all analytes ranged from 2.61 to 5.94 min as presented in Table 1 and remained stable throughout measurements. An overview of the chromatographic separation is presented in Fig. 2 where EICs of the five targeted ions corresponding to the analytes measured as standard solutions are collected. In Fig. 3, EICs of the analytes are represented in completely blank matrix (A), in blank matrix spiked with ISTDs (B), a typical QC sample at the 0.5 ng/mL concentration as well as inside three authentic samples (D-F). Even at low concentrations, well-defined peaks were found with the exception of the two 5-DHP isomers, which partially co-eluted at 5.81 and 5.94 min, and P5 and 3β-DHP, which overlapped at 3.52 min. These two pairs of isobaric steroids vary only in the position of a double bond and are therefore structurally greatly similar. All other analytes were successfully separated by means of chromatography or their ions had different masses. No serious interferences were found in the blank plasma at the retention time of all analytes and the ISTDs. A high resolution of 35,000 was chosen in order to observe an appropriate baseline level in the chromatograms.

3.2.2. Carry-over

Peak areas of pure MeOH and blank plasma samples were investigated after a previous injection of a 1 μg/mL standard solution, a concentration that is at least 100 times higher than to be expected in an authentic sample. Small peaks were found in the blank solvent for the compounds 20α-DHP, 20β-DHP and P4 as they are readily ionizable. However, their area fulfilled the criteria to be below 20% of the peak area of the LOQ sample. Concerning the plasma blank, no carry-over was determined. The minimal carry-over observed in the blank solvent most likely vanished in the higher background noise.

3.2.3. Limit of detection, limit of quantification and standard calibration curves

The limit of detection ranged from 0.005 to 0.1 ng/mL, whereas the limit of quantification was determined as 0.02–0.3 ng/mL (Table 2). Thereby, same values were obtained for diastereoisomers due to their similar ionizability with the exception of the 3-DHP compounds as well as 3β,5β-THP. Concerning the 3-DHP derivatives, 3β-DHP was coeluting with P5 and its detection limit is hence overestimated (0.02 ng/mL) and lower than of 3α-DHP (0.05 ng/mL). In contrast to this, the limit of quantification was three times higher for 3β,5β-THP (0.3 ng/mL) compared with the remaining tetrahydroprogestagens (0.1 ng/mL). This higher limit of quantification had to be set after the measurement of the compound in authentic plasma samples. Compared to charcoal stripped plasma, authentic samples own a more complex matrix and thus, ion suppression was sometimes an issue for the 3β,5β-THP steroid at lower concentrations. To ensure reliable values even for authentic samples, the LOQ of 3β,5β-THP was increased. The lowest LOD and LOQ could be attributed to the steroids P4, 20α- and 20β-DHP that all are characterized by a conjugated ketone. Because of these different sensitivities, 2 ISTD were taken. P4-*d9* was chosen for the compounds with lowest LOD, whereas P5-*d4* was suitable for the less ionizable compounds (3α,5α-THP, 3α,5β-THP, 3β,5α-THP, 3β,5β-THP, 3α-DHP, 3β-DHP, 5α-DHP, 5β-DHP and P5). Nevertheless, the overall sensitivity was adequate to investigate biological systems where steroid hormones are often produced at only low quantities in the range of ng/mL or even pg/mL. The correlation coefficients for the regression equations were generated for each measurement sequence (n = 4) and were always higher than 0.99 indicating a very good, reproducible fit.

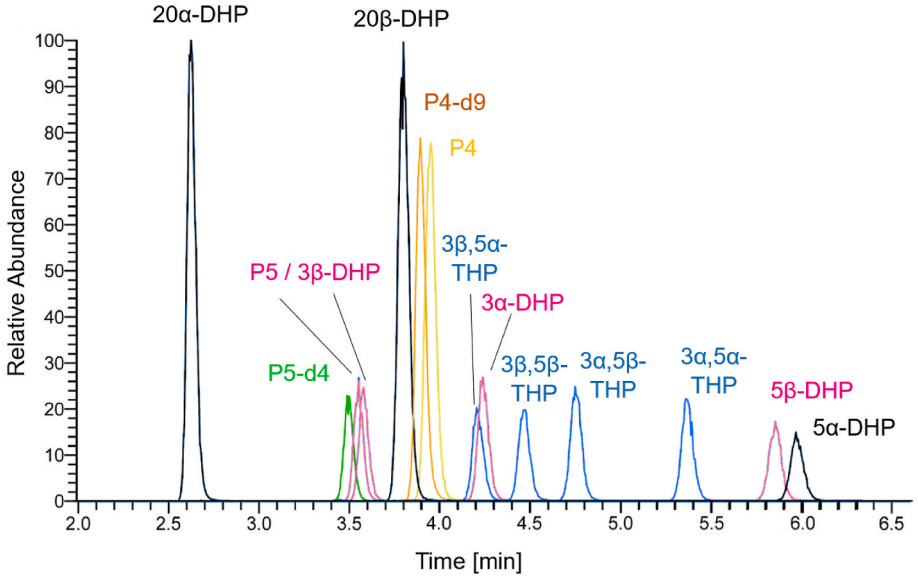

Fig. 2. Overlaid extracted ion chromatograms (±5 ppm) of quantifying ions (see Table 1) measured from standard solutions of equal concentration.

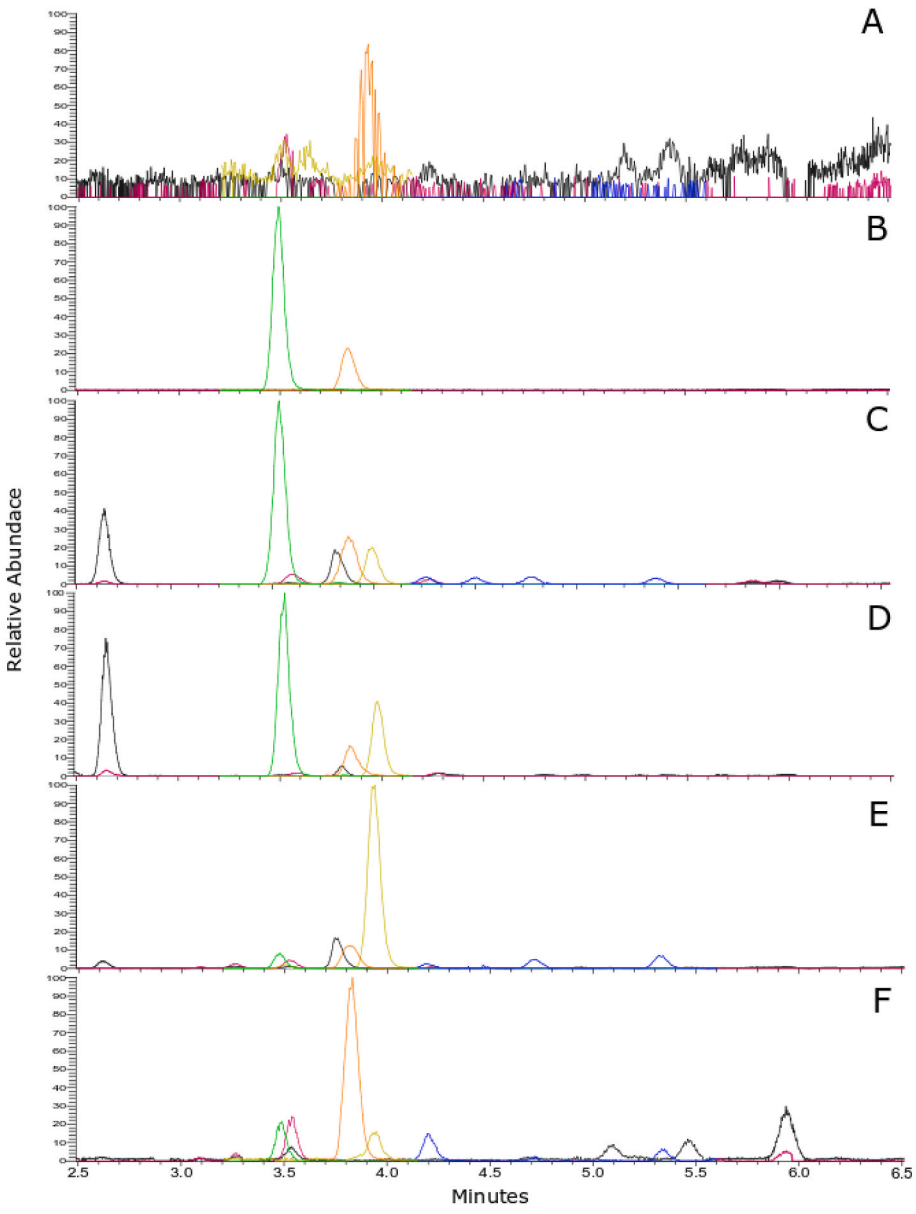

Fig. 3. Overlaid extracted ion chromatograms (±5 ppm) of quantifying ions (see Table 1) of authentic samples measured by UHPLC-MS using msx t-SIM (multiplexed targeted single ion monitoring) in the relevant time range between 2.5 and 6.5 min. (A) Charcoal treated plasma sample; (B) blank plasma sample spiked with both ISTDs; (C) representative 0.5 ng/mL QC sample; (D) authentic plasma sample of a roe deer (*Capreolus capreolus*); (E) authentic plasma sample of a cow (*Bos taurus*); (F) authentic serum sample of an Asian elephant (*Elephas maximus*).

Table 2
Limits of detection and quantifications for all steroid hormones together with the smallest value obtained for the correlation coefficient comparing all calibration curves (n = 4).

Analyte	LOD (ng/mL)	LOQ (ng/mL)	R^2 (a.u.)
20α-DHP	0.005	0.02	≥0.9998
20β-DHP	0.005	0.02	≥0.9998
3α-DHP	0.05	0.1	≥0.9933
3β-DHP	0.02	0.05	≥0.9988
5α-DHP	0.1	0.2	≥0.9962
5β-DHP	0.1	0.2	≥0.9969
3α,5α-THP	0.05	0.1	≥0.9982
3α,5β-THP	0.05	0.1	≥0.9996
3β,5α-THP	0.05	0.1	≥0.9987
3β,5β-THP	0.05	0.3	≥0.9996
P4	0.005	0.05	≥0.9989
P5	0.02	0.05	≥0.9988

Least squares regression analysis yielded the result of a quadratic fitting model with an equal weighting for the ratio of the peak area of the analyte and its ISTD against the nominal concentration. The curve was forced through the origin to obtain more accurate values at low concentrations. Illustrated in Fig. 4, a large dynamic range can be observed for all analytes up to 240 ng/mL from which point on the curve begins to flatten. This non-linearity at high concentrations is common for ESI measurements and can be explained by ionization and/or detector saturation as well as the formation of dimer/cluster ions [23].

3.2.4. Accuracy and precision

Accuracy and precision of the method were evaluated at three different concentration levels of QC samples in duplicate on four different days. The QC levels (between 0.06 and 2 ng/mL) were chosen to cover the most relevant biological range expected in authentic samples to ensure high data quality. The accuracy is given as a relative error (RE, %), whereas intra- and inter-day precision were calculated as a relative standard deviation (RSD, %). The results are summarized in Table 3. The relative error of the accuracy ranged from −12.3% to 10.4% for all compounds. The precision was very good for most progestagens with RSD values between 1.1% and 15.7%. Only 3α-DHP shows a higher precision deviation up to 17.4% and is thus slightly above our criteria to have a precision below 15% at all concentrations.

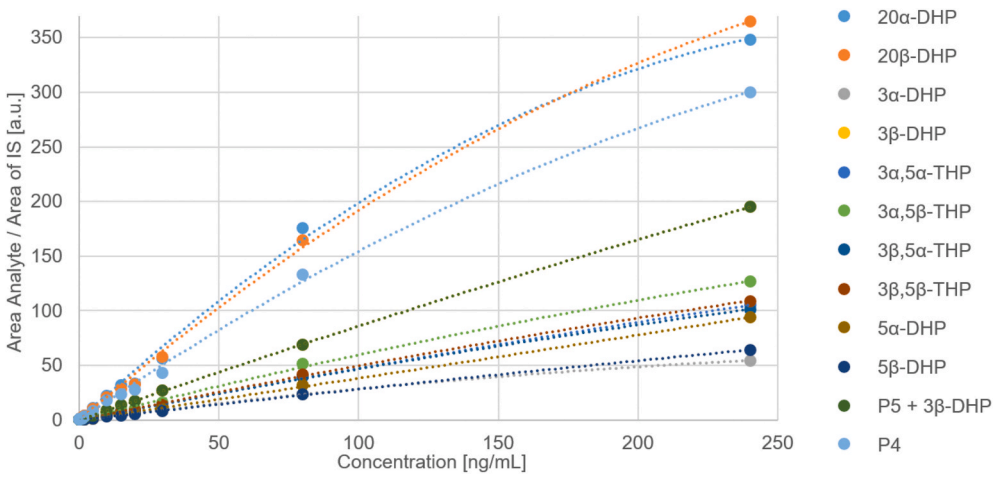

Fig. 4. Exemplary calibration curves of all analytes based on a quadratic fit and forced through the origin.

Table 3
Calculated validation results including accuracy (bias), intra- (RSD_R) and inter- (RSD_T) day precision (n = 8).

Analyte	QC level	Concentration (ng/mL)	Mean calculated concentration (ng/mL)	Bias (%)	RSD_R (%)	RSD_T (%)
20α-DHP	low	0.06	0.058	−2.7	6.8	5.5
	middle	0.5	0.524	4.8	4.0	5.1
	high	2.0	1.943	−2.9	5.1	6.7
20β-DHP	low	0.06	0.061	0.8	3.0	3.5
	middle	0.5	0.506	1.1	2.9	3.7
	high	2.0	1.910	−4.5	5.2	8.5
3α-DHP	low	0.2	0.175	−12.3	17.3	14.9
	middle	0.5	0.505	1.0	13.0	10.7
	high	2.0	1.872	−6.4	13.0	17.4
3β-DHP	low	0.06	0.066	10.4	6.9	8.2
	middle	0.5	0.518	3.6	3.1	3.7
	high	2.0	1.890	−5.5	5.0	5.2
5α-DHP	low	0.2	0.222	11.1	8.9	9.3
	middle	0.5	0.510	2.0	4.0	4.1
	high	2.0	1.915	−4.2	5.0	7.1
5β-DHP	low	0.2	0.204	1.9	8.0	6.3
	middle	0.5	0.510	1.9	1.4	2.2
	high	2.0	2.011	0.6	6.7	6.3
3α,5α-THP	low	0.2	0.198	−1.1	5.2	7.1
	middle	0.5	0.497	−0.5	1.5	4.0
	high	2.0	1.955	−2.3	4.4	5.2
3α,5β-THP	low	0.2	0.185	−7.3	3.7	15.7
	middle	0.5	0.518	3.7	1.1	2.9
	high	2.0	1.984	−0.8	3.2	7.7
3β,5α-THP	low	0.2	0.198	−2.6	4.5	11.3
	middle	0.5	0.510	2.0	3.1	3.4
	high	2.0	1.894	−5.3	5.2	7.1
3β,5β-THP	low	0.4	0.400	−0.1	4.5	4.9
	middle	0.5	0.515	3.0	4.9	4.1
	high	2.0	1.939	−3.1	1.5	4.0
P4	low	0.06	0.063	5.2	4.0	5.7
	middle	0.5	0.505	1.0	4.0	3.0
	high	2.0	1.876	−6.2	5.7	7.2
P5	low	0.06	0.066	10.4	6.9	8.2
	middle	0.5	0,519	3.6	3.1	3.7
	high	2.0	1.890	−5.5	5.0	5.2

3.2.5. *Recovery and matrix effect*

In order to investigate the extraction efficiency of the SPE procedure over a broad range, recovery and matrix effects were investigated using three-levelled steroid concentrations as listed in Table 4. Relative standard deviations were calculated from the mean of six measurements for each sample. In plasma samples, the recovery at the low concentration of 0.4 ng/mL was above 50% in all cases. For the middle and high concentration levels, the recovery lied between 62.8% and 109.6%. Matrix effects beneath 100% indicate ion suppression, whereas values above 100% refer to ion enhancement. For 20β-DHP, the most ion suppression was observed at the low concentration (0.4 ng/mL) with 44.3%. However, little matrix effects were overall noted. For example, at high concentrations, the matrix effect was between 63.4% and 105.1%. Thus, only little amounts are lost during the SPE purification and most of the possible interferents were removed efficiently.

3.3. *Applicability*

3.3.1. *Progestogen profiling in plasma of cyclic cattle (Bos taurus)*

Progestogens in 145 plasma samples of five different cows were successfully quantified by our established method. Due to the method's high sensitivity, several progestogens were detected and monitored in a

Table 4
Calculated recoveries and matrix effects together with their relative standard deviations (n = 6).

Analyte	QC level	Nominal concentration (ng/mL)	Recovery (%)	RSD (%)	Matrix effect (%)	RSD (%)
20α-DHP	low	0.4	62.3	30.0	73.6	34.4
	middle	6.0	94.5	32.4	85.4	37.8
	high	18.0	99.0	23.4	98.2	32.4
20β-DHP	low	0.4	64.2	30.1	44.3	37.9
	middle	6.0	98.2	29.4	51.5	35.2
	high	18.0	102.2	20.7	63.4	29.4
3α-DHP	low	0.4	54.7	14.1	57.5	18.9
	middle	6.0	62.8	26.1	77.7	18.8
	high	18.0	83.9	2.6	86.8	26.1
3β-DHP	low	0.4	73.6	13.8	71.1	7.5
	middle	6.0	82.2	17.5	82.2	11.6
	high	18.0	109.6	6.4	82.0	17.5
5α-DHP	low	0.4	70.7	7.8	91.5	14.3
	middle	6.0	75.8	7.9	97.1	6.2
	high	18.0	89.0	6.7	105.1	7.9
5β-DHP	low	0.4	73.2	4.2	92.8	7.8
	middle	6.0	85.9	6.6	86.9	8.1
	high	18.0	91.6	7.6	99.3	6.6
3α,5α-THP	low	0.4	76.2	3.3	83.3	6.6
	middle	6.0	79.4	4.8	92.3	5.4
	high	18.0	95.6	6.5	95.6	4.8
3α,5β-THP	low	0.4	78.1	4.3	81.5	11.2
	middle	6.0	82.7	5.7	89.6	7.4
	high	18.0	98.7	7.4	95.9	5.7
3β,5α-THP	low	0.4	74.3	7.5	84.1	7.8
	middle	6.0	80.6	4.2	93.5	5.6
	high	18.0	96.7	5.1	97.4	4.2
3β,5β-THP	low	0.4	76.5	5.8	83.2	11.6
	middle	6.0	83.1	6.1	91.0	8.0
	high	18.0	98.2	6.6	95.5	6.1
P4	low	0.4	62.8	25.6	57.1	34.8
	middle	6.0	92.8	27.6	64.2	35.2
	high	18.0	100.1	24.8	71.8	27.6
P5	low	0.4	73.6	13.8	71.1	7.5
	middle	6.0	82.2	17.5	82.2	11.6
	high	18.0	109.6	6.4	82.0	17.5

time-resolved fashion over the course of multiple weeks giving valuable insight in the reproductive biology in cattle. The corresponding data and their interpretation have been published by Hankele et al. [24].

3.3.2. Progestogen profiling in plasma of female roe deer (Capreolus capreolus)

Over 100 plasma samples of individual roe deer have also been analyzed with this method with a focus on the course of embryonic diapause in the European roe deer. Again, several progestagens were detected and quantified. The data are described in more detail in the publication of van der Weijden et al. [25].

3.3.3. Progestogen profiling in serum of pregnant Asian elephants (Elephas maximus)

The applicability of our novel method was further proven by the measurement of progestogens in 130 serum samples of pregnant Asian elephants. Contrary to most mammals, the most abundant circulating progestogen in elephants is not P4 but its 5α reduced form [26]. Since our method allows the detection and quantification of both, P4 and its reduced metabolites, it is highly suitable for studying gestagens in female elephants. We were able to successfully quantify several progestagens as well as the low amounts of progesterone in the serum of pregnant elephants over the course of pregnancy until parturition (data not shown).

4. Conclusion

A functional and high-throughput UHPLC-HRMS protocol based on a Q Exactive mass spectrometer was established and validated for the simultaneous quantification of twelve progestogens (20α-DHP, 20β-DHP, 3α,5α-THP, 3α,5β-THP, 3β,5α-THP, 3β,5β-THP, 3α-DHP, 3β-DHP, 5α-DHP, 5β-DHP, P5, P4) in plasma and serum with a run time of only 14 min per sample. A purification process was developed by an optimized SPE method without the need for a labor-intensive derivatization step. The instrumental method was based on UHPLC-HR-MS employing ESI ionization and multiplexed t-SIM that achieved a high sensitivity in the range of pg/mL as well as a good selectivity sufficient for biological systems. All validation tests were successfully conducted and showed that charcoal stripped bovine plasma is a first-rate choice as a surrogate matrix. Accuracy tests resulted in adequate values within ±13%. The intra- and interday precision were determined as being under 17.4%. The applicability was proven by the quantification of progestogens in cattle and roe deer plasma as well as elephant serum samples. Thus, this quantification method can be easily applied for routine analyses in various species including humans.

Funding

This study was funded in part by the Swiss National Science Foundation SNSF (206021_183310 and 310030_185026).

Declaration of competing interest

All authors declare that no conflict of interest is present regarding this work.

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